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☐ *Additional inventors are being named on the ___ separately numbered sheets attached hereto*

TITLE OF THE INVENTION (280 characters max)

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ENCLOSED APPLICATION PARTS *(check all that apply)*

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Respectfully submitted,

SIGNATURE _____ Date 3 / 24 /99

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PROVISIONAL APPLICATION

UNDER 37 CFR 1.53(b)(2)

TITLE: **CONTINUOUS SURFACE ARRAYS**

APPLICANT: **DAVID ENGLERT, GIRISH NALLUR AND ALICIA HULBERT**

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CONTINUOUS SURFACE ARRAYS

Background of the Invention

Rapid advances in the ability to accurately determine polynucleotide sequences, such as DNAs and RNAs from the genomes of organisms, has made possible the sequencing of huge quantities of polynucleotides. In recent years, the entire genomes of microorganisms, such as Helicobacter pylori, have been sequenced.

Traditional sequencing methods have relied on automated sequencing equipment which processes a polynucleotide strand one base at a time. A more recent approach, sequencing by hybridization (SBH), which could potentially increase sequencing throughput, relies on fragmenting a target polynucleotide into short segments; these short segments can be captured, for example on an ordered array of immobilized complementary strands, and the sequences of the individual fragments determined. Alignment of the fragment sequences, typically with the aid of a computer for longer sequences of interest, provides the sequence of the target polynucleotide. See, for example, U.S. Patent No. 5,552,270 to Khrapko et al.

Arrays of immobilized polynucleotides have been constructed for use in SBH techniques. However, new types of arrays, and methods for making them, are needed.

Summary of the Invention

The invention features arrays of a molecules, e.g., polymeric molecules such as nucleic acids and polypeptides, on a continuous surface, e.g., a surface of a polyacrylamide or agarose gel, and methods for making and using the continuous surface arrays. By "continuous" is meant a planar surface substantially free of indentations or

impressions. The arrays can be used for sequencing by hybridization (e.g., where the arrays include nucleic acid strands immobilized to the gel), for cell based assays (e.g., where the arrays include, or are adjacent to and contacting, living cells), and for other uses which will be apparent to one of ordinary skill in the art.

In one aspect, the invention provides a method for preparing an array of polymers having diverse sequences on a continuous surface, e.g., a surface of a polyacrylamide or agarose gel. The method includes

(a) providing a substrate comprising a continuous surface;

(b) applying a first reagent comprising a monomer to a first locus on said surface;

(c) displacing said single reagent dispenser relative to said surface;

(d) applying a second reagent comprising a second monomer to said surface; and

(e) repeating steps (b)-(d) until an array of polymers having diverse sequences are formed.

In preferred embodiments, the substrate is a continuous film, e.g., a polyacrylamide or agarose gel substrate.

This method can be used to form a two dimensional array of probes, wherein each of the probes is positionally distinguishable from other probes of the plurality on the array, and wherein each positional distinguishable probe includes a unique (i.e., not repeated in another probe) region.

The array density, i.e., the number of spatially distinct addresses on the substrate, is at least 10, more preferably 100, more preferably 1,000, or even more

preferably 10,000, pores per square centimeter of continuous membrane surface.

A substance can be deposited on the array with a piezo dispensing device.

In another aspect, the invention features a two dimensional array of molecules wherein each of the molecules is positionally distinguishable from other molecules of the plurality on the array, and wherein each positional distinguishable molecules is different form the others. The molecules can be nucleic acids, e.g., DNA or RNA molecules, polypeptides, or polysaccharides. Nucleic acid molecules will preferably have a unique (i.e., not repeated in another nucleic acid of the plurality) region. The array is disposed on a continuous film substrate as described herein. Such arrays are referred to herein as continuous surface arrays, continuous film arrays, two dimensional arrays, or simply arrays.

In another aspect, the invention features a method of making a two dimensional array of molecules on a continuous surface wherein each of the molecules is positionally distinguishable from other molecules of the plurality on the array, and wherein each positional distinguishable molecules is different form the others. The molecules can be polymers of nucleic acids such as DNA or RNA, or polymers of amino acids, e.g., oligonucleotides, oligopeptides, nucleic acids, or polypeptides. Nucleic acid molecules will preferably have a unique (i.e., not repeated in another nucleic acid of the plurality) region. The array is disposed on a continuous film surface as described herein.

In another aspect, the invention features a method of analyzing a test nucleotide in a polynucleotide, e.g.,

detecting a genetic event, e.g., a single nucleotide polymorphism, in a sample.

The method includes:

(1) providing a sample which includes a sample polynucleotide sequence, which includes a test nucleotide, to be analyzed;

(2) (a) annealing an effective amount of sample polynucleotide sequence to a single-stranded circular template, wherein the single-stranded circular template includes at least one copy of a nucleotide sequence complementary to the sequence of the sample polynucleotide sequence and optionally,

(b) combining the circular template with an effective amount of at least two types of nucleotide triphosphates and an effective amount of a polymerase enzyme to yield a product, e.g., a single-stranded oligonucleotide multimer complementary to the circular oligonucleotide template; and optionally

cleaving the product to produce cleaved amplified product,

(3) analyzing said product from (2) b or c, e.g., by providing a two dimensional continuous surface array, e.g., such as is described herein, e.g., one having of a plurality of capture probes, wherein each of the capture probes is positionally distinguishable from other capture probes of the plurality on the array, and wherein each positional distinguishable capture probe includes a unique (i.e., not repeated in another capture probe) region;

hybridizing the product with the array of capture probes, thereby analyzing the sample sequence.

In preferred embodiments the capture probes are single stranded probes in an array.

In a preferred embodiment the test nucleotide, or its complementary nucleotide, is sufficiently close to an

end of the cleaved amplified product such that its presence can be detected by its effect on a reaction which involves the end nucleotide of the cleavage product.

In preferred embodiments, the sample nucleic acid serves as a primer. In other preferred embodiments synthesis is primed with a nucleic acid other than the sample nucleic acid.

In preferred embodiments, the reaction is a hybridization reaction. In other preferred embodiments, the reaction is a ligation reaction, a polymerization reaction, e.g., a DNA polymerase catalyzed reaction, modification, or a restriction or other cleavage reaction.

In preferred embodiments, the test nucleotide, or its complementary nucleotide, is within 1 (i.e., it is at the end), 2, 3, 4 or 5 base pairs from the end of the cleaved amplified product.

Numerous variations in the circular oligonucleotide template are within the invention. As is described herein, the circular oligonucleotide template can be formed, e.g., in the reaction mix, from a linear precursor. As is described herein, the circular oligonucleotide can be resistant to cleavage, providing for more efficient amplification. Other variants are described below.

In preferred embodiments, the circular oligonucleic acid template is prepared by a process which includes the steps of:

(a) hybridizing each end of a linear precursor oligonucleotide to a positioning oligonucleotide, e.g., a sample sequence, (wherein the positioning oligonucleotide has a 5' nucleotide sequence complementary to a portion of the sequence which includes the 3' end of the linear precursor oligonucleotide and a 3' nucleotide sequence complementary to a portion of the sequence which includes

the 5' end of the linear precursor oligonucleotide, to yield an open oligonucleotide circle wherein the 5' end and the 3' end of the open circle are positioned such that they can be joined, e.g., as to abut each other; and

(b) joining the 5' end and the 3' end of the open oligonucleotide circle to yield a circular oligonucleotide template. Rolling circle amplification can be primed by the positioning oligonucleotide, e.g., the target nucleic acid, or by another in this or other methods disclosed herein.

In preferred embodiments, the oligonucleotide multimer is more sensitive to cleavage than is the circular template. In preferred embodiments the oligonucleotide multimer is cleaved and the circular template is not cleaved. For example, in preferred embodiments the circular template has one or more nucleotide or modified nucleotide which is resistant to cleavage. While the modified nucleotide is resistant to cleavage, when it serves as a template, it nevertheless incorporation of the appropriate complementing nucleotide. The circular template, but preferably not the oligonucleotide multimer, can include one or more of deoxy uracil, or a methylated or hemimethylated base to render the circular template more resistant to cleavage.

Preferential cleavage of the oligonucleotide multimer can be effected by hybridization of a cleavage probe (thus forming a double stranded cleavage site) and said cleavage probe is chosen such that it cannot displace a strand from the circular template, thus allowing cleavage of only the oligonucleotide multimer.

Preferably, a circular template has about 15-1500 nucleotides, and more preferably about 24-500 nucleotides and most preferably about 30-150 nucleotides.

The oligonucleotide circular template itself may be constructed of DNA or RNA or analogs thereof. Preferably, the circular template is constructed of DNA. A liquid, e.g., a sample nucleic acid or protein binds to a portion of the circular template and is preferably single-stranded having about 4-50 nucleotides, and more preferably about 6-12 nucleotides.

In preferred embodiments the circular template includes a site for a type 2S restriction enzyme and the site is positioned, e.g., such that a type 2S restriction binding at the site cleaves adjacent the region which binds the sample sequence, cleaves in the region which binds the sample sequence, or cleaves at the target nucleotide.

In a preferred embodiment a region of the circular template is complementary to a genetic event, e.g., a mutation or SNP, and hybridizes differentially to a sample nucleic acid having the event and sample nucleic acid not having the event.

In preferred embodiments the reactions in one or more, and preferably all of steps 1, 2, and 3 are performed at the same temperature.

In preferred embodiments the reactions in one or more of steps 1, 2, and 3 are performed in the same container.

In preferred embodiments, analyzing a sample polynucleotide sequence includes, e.g., sequencing at least one nucleotide of the polynucleotide sequence, e.g., by sequencing by hybridization or positional sequencing by hybridization, detecting the presence of, or identifying, a genetic event, e.g., a SNP, in a target nucleic acid, e.g., a DNA.

In preferred embodiments, the genetic event is within 1, 2, 3, 4 or 5 base pairs from the end of the target

molecule, or is sufficiently close to the end of the target molecule that a mismatch would inhibit DNA polymerase-based extension from a target/ primed circle.

In preferred embodiments, the target nucleic acid is amplified, e.g., by PCR, prior to contact with a circular template.

In preferred embodiments the polynucleotide sequence is: a DNA molecule: all or part of a known gene; wild type DNA; mutant DNA; a genomic fragment, particularly a human genomic fragment; a cDNA, particularly a human cDNA.

In preferred embodiments the polynucleotide sequence is: an RNA molecule: nucleic acids derived from RNA transcripts; wild type RNA; mutant RNA, particularly a human RNA.

In preferred embodiments the polynucleotide sequence is: a human sequence; a non-human sequence, e.g., a mouse, rat, pig, primate.

In preferred embodiments the method is performed: on a sample from a human subject; and a sample from a prenatal subject; as part of genetic counseling; to determine if the individual from which the target nucleic acid is taken should receive a drug or other treatment; to diagnose an individual for a disorder or for predisposition to a disorder; to stage a disease or disorder.

The products of rolling circle amplification can be analyzed in various ways. In some embodiments, the products of rolling circle application are analyzed on positional arrays.

Accordingly, in preferred embodiments the method further includes:

(3) analyzing said product from (2) b or c, e.g., by providing an array of a plurality of capture probes, wherein each of the capture probes is positionally distinguishable

from other capture probes of the plurality on the array, and wherein each positional distinguishable capture probe includes a unique (i.e., not repeated in another capture probe) region;

hybridizing the product with the array of capture probes, thereby analyzing the sample sequence.

In preferred embodiments, the array is a continuous film array as described herein.

In preferred embodiments the capture probes are single stranded probes in an array.

In preferred embodiments the capture probes have a structure comprising a double stranded portion and a single stranded portion in an array.

In preferred embodiments hybridization to the array is detected by mass spectrophotometry, e.g., by MALDI-TOF mass spectrophotometry.

In a preferred embodiment the amplified sample sequence which hybridizes to a capture probe, or the capture probe, is the substrate of or template for an enzyme mediated reactions.

For example, after hybridization to the capture probe, the amplified sample sequence is ligated to the capture probe, or after hybridization it is extended along the capture probe.

In preferred embodiments the method includes one or more enzyme mediated reactions in which a nucleic acid used in the method, e.g., an amplified sample sequence, a capture probe, a sequence to be analyzed, or a molecule which hybridizes thereto, is the substrate or template for the enzyme mediated reaction. The enzyme mediated reaction can be: an extension reaction, e.g., a reaction catalyzed by a polymerase; a linking reaction, e.g., a ligation, e.g., a

reaction catalyzed by a ligase; or a nucleic acid cleavage reaction, e.g., a cleavage catalyzed by a restriction enzyme, e.g., a Type IIs enzyme. The amplified sample sequence which hybridizes with the capture probe can be the substrate in an enzyme mediated reaction, e.g., it can be ligated to a strand of the capture probe or it can be extended along a strand of the capture probe. Alternatively, the capture probe can be extended along the hybridized amplified sample sequence. (Any of the extension reactors discussed herein can be performed with labeled, or chain terminating, subunits.) The capture probe duplex can be the substrate for a cleavage reaction. These reactions can be used to increase specificity of the method or to otherwise aid in detection, e.g., by providing a signal.

Methods of U.S. Patent No. 5,503,980 and or U.S. Patent No. 5,631,134, both of which are hereby incorporated by reference can be used herein, particularly, the array and array-related steps recited herein can use methods taught in these patents.

In preferred embodiments, the method includes providing an array having a plurality of capture probes, wherein each of the capture probes is a) positionally distinguishable from the other capture probes of the plurality and has a unique variable region (not repeated in another capture probe of the plurality), b) has a variable region capable of hybridizing adjacent to the genetic event; and has a 3' end capable of serving as a priming site for extension;

hybridizing the amplified sample sequence having a genetic event to a capture probe of the array, (preferably the region of the amplified sample sequence having a genetic event hybridizes adjacent to the variable region of a capture probe); and

using the 3' end of the capture probe to extend across the region of genomic nucleic acid having a genetic event with one or more terminating base species, where if more than one is used each species has a unique distinguishable label e.g. label 1 for base A, label 2 for base T, label 3 for base G, and label 4 for base C; thereby analyzing the amplified sample sequence.

In a preferred embodiment, at least one reaction step is performed on the dimensional continuous film array, as described herein. In preferred embodiments the method includes providing a plurality of single-stranded circular templates, wherein each of the single-stranded circular templates is positionally distinguishable from other single-stranded circular templates of the plurality on the array, and wherein each positional distinguishable single-stranded circular templates includes a unique (i.e., not repeated in another circular template) region complementary to sample target.

The polymerase enzyme can be any that effects the synthesis of the multimer, e.g., any polymerase described in U.S. Pat. No. 5,714,320. Generally, the definitions provided for circular vectors and their amplification in U.S. Patent No. 5,714,320, apply to terms used herein, unless there is a conflict between the terms in which case the meaning provided herein controls. U.S. Patent No. 5,714,320 and all other U.S. patents mentioned herein are incorporated by reference.

In preferred embodiments the primer, the sample, or the product is immobilized.

In preferred embodiments, the method further includes providing an effective amount of a cleavage primer which can hybridize to the oligonucleotide multimer, wherein the cleavage primer has at least one copy of a cleavage site

flanked by a first and second detection moiety, and wherein the second detection moiety affects the signal produced by the first moiety and upon cleavage at the cleavage site, the distance between the two moieties increases, resulting in an alteration of the signal, and cleaving the oligonucleotide multimer at the cleavage site to produce the cleaved amplified product;

and (3) analyzing said product from (2) b or c, e.g., thereby analyzing the sample sequence.

In preferred embodiments, the signal increases upon cleavage at the cleavage site.

In other preferred embodiments, the signal decreases upon cleavage at the cleavage site.

In preferred embodiments, the method further includes providing an effective amount of a first detection oligonucleotide having a first detecting moiety and a second oligonucleotide having a second detecting moiety, wherein the first and second oligonucleotides hybridize to the oligonucleotide multimer so that upon hybridization the first detection oligonucleotide hybridizes sufficiently close to the second detection oligonucleotide, such that the second detection moiety affects the signal produced by the first detection moiety.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the

present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

Detailed Description

Embodiments of the invention are based on the use of continuous film arrays of the invention to analyze a sequence, e.g., to sequence the nucleic acid in question, or to identify SNPs, mutations and RNA molecules, or to clarify a sample, e.g., as to disease state, or generally in expression profiling or analysis. The analyses are performed using circular vectors (as described in U.S. Patent No. 5,714,320). The circular vectors can be closed circular vectors, open circular vectors which when brought into contact with the analyte, have abutting ends which can be covalently linked, e.g., ligated.

Construction of Continuous Film Arrays

In general, any method known in the art that is capable of generating arrays having spatially distinguishable molecules. Continuous film arrays are preferably constructed by using a piezo-dispensing device, e.g., the device described in USSN 08/656,455.

Piezo-electric dispensing allows for a small volume, e.g., 350 picoliters, to be dispensed into each array position. Small volumes result in small spot sizes in the two dimensional arrays as projected onto a plane parallel to the support of the porous matrix, because the dispensed sample occupies a very small volume when applied to the porous matrix. Small spot sizes are important for achieving

a high density of molecules, e.g., probe molecules, in the array.

The two dimensional array may be disposed within a porous three-dimensional matrix, which is preferably a polymer matrix that is supported by wither a solid or porous substrate.

In general, any chemistry that results in immobilization of a molecule to be used to construct an array, e.g., DNA, RNA, proteins, or carbohydrates, and that prevents significant diffusion out of or thin the matrix before immobilization occurs are suitable for creating high density of probe molecules. Methods for attaching monomers such as nucleotides are known in the art and are described, e.g., in Proudnikov et al., Anal. Biochem. 259:34-41, 1998; Timofeev et al., NAR 24:3142-48, 1996, and Yershov et al. Proc. Nat. Acad. Sci (USA) 93:4913-4918, 1996. These chemistries include reacting active esters, such as N-Hydroxy-succinimdyl esters or isothiocyantes on probe molecules with amine-containing matrices. Alternatively, corresponding photoreactive groups on the probe and gel matrix molecules can be used. For example, psoralen can be incorporated into the gel matrix, and ultraviolet light can be used to bind the nucleic acid probe molecules to the psoralen-modified gel matrix. A focused beam of ultraviolet light can be focused on the matrix immediately after dispensing the oligonucleotide to effect immobilization before the probe molecules diffuse significantly within the gel matrix. Another method for attaching molecules to the gel matrix is by incorporating ligand molecules such as strepavidin into the gel matrix. Addition of a nucleic acid probe molecule results in formation of stable, but non-covalent, attachment of the nucleic acid molecule to the matrix.

Rolling Circle Amplification

Rolling circle amplification (RCA) is used to generate many copies of an oligonucleotide preferably with defined ends (as described in U.S. Pat. No. 5,714,320). The single-stranded product of rolling circle amplification can be rendered double-stranded by the annealing of un-circularized, complementary probe vector. The dsDNA RCA product can be fragmented, e.g., using a type IIS restriction enzyme, such that the DNA is cleaved in the middle, or at the ends, of the region generated by the ligation reaction. The dsDNA fragments generated by the restriction digest can be analyzed, e.g., on an array, e.g., an array of indexing linkers (see, e.g., U.S. Patent No. 5,508,169). If the probe vector is labeled with capture or anchoring moiety, e.g., a biotin group, then it is possible to render the dsDNA fragments generated from fragmentation of the RCA product single-stranded by thermal denaturation following the addition of capture or anchoring moiety reactive, e.g., strepavidin-labeled, substrates, e.g., magnetic beads or a solid support. The single-stranded DNA fragments can be analyzed on a Cantor array. These oligonucleotides are analyzed on a Cantor Capture array (see, e.g., 5,503,980) using, e.g., fluorescent detection methodology.

In other embodiments, the captured DNA fragments are analyzed using mass spectrometry. Alternatively, the target DNA is applied to a multiplicity of wells and a population of RCA vectors is added to each well. The RCA products are analyzed using mass spectrometry following fragmentation, where the amplification of specific RCA vectors is determined by differences in molecular weight of the RCA product fragments. Multiple RCA vectors can be analyzed simultaneously in a single reaction using this approach.

Positional Arrays

Positional arrays suitable for the present invention include high and low density arrays made on the continuous film arrays. Positional arrays include nucleic acid molecules, peptide nucleic acids or high affinity binding molecules of known sequence attached to predefined locations on a surface. Arrays described in numerous patents which are incorporated herein by reference, Cantor, US Patent No.,5,503,980; Southern EP 0373 203 B1; Southern, U.S. Patent No. 5,700,637, and Deugau, U.S. Patent No. 5,508,169, can be adapted to continuous film arrays of the invention. The density of the array can range from a low density format a 96- or 384- array matrix, to a high density format, e.g. 1000 molecules/cm2, as described in Fodor US 5,445,934.

In preferred embodiments, the target or probes bind to (and can be eluted from) the array at a single temperature. This can be effected by manipulating the length or concentration of the array or nucleic acid which hybridizes to it, by manipulating ionic strength or by providing modified bases.

Proximity Methods

Proximity methods include those methods whereby a signal is generated when a first member and second member of a proximity detection pair are brought into close proximity.

A "proximity detection pair" will have two members, the first member, e.g., an energy absorbing donor or a photosensitive molecule and the second member, e.g., an energy absorbing acceptor or a chemiluminescer particle. When the first and second members of the proximity detection pair are brought into close proximity, a signal is generated. Examples of proximity methods include the following:

Fluorescence resonance energy transfer (FRET)

Fluorescence resonance energy transfer (FRET) is based on a donor fluorophore that absorbs a photon of energy and enters an excited state. The donor fluorophore transfers its energy to an acceptor fluorophore when the two fluorophores are in close proximity by a process of non-radiative energy transfer. The acceptor fluorophore enters an excited state and eliminates the energy via radiative or non-radiative processes. Transfer of energy from the donor fluorophore to acceptor fluorophore only occurs if the two fluorophores are in close proximity.

Homogeneous time resolved fluorescence (HTRF)

Homogeneous time resolved fluorescence (HTRF) uses FRET between two fluorophores and measures the fluorescent signals from a homogenous assay in which all components of the assay are present during measurement. The fluorescent signal from HTRF is measured after a time delay, thereby eliminating interfering signals. One example of the donor and acceptor fluorophores in HTRF include europium cryptate [(Eu)K] and XL665, respectively.

Luminescent oxygen channeling assay (LOCI)

In the luminescent oxygen channeling assay (LOCI), the proximity detection pairs includes a first member which is a sensitizer particle that contains phthalocyanine. The phthalocyanine absorbs energy at 680 nm and produces singlet oxygen. The second member is a chemiluminescer particle that contains olefin which reacts with the singlet oxygen to produce chemiluminescence which decays in one second and is measured at 570 nm. The reaction with the singlet oxygen and the subsequent emission depends on the proximity of the first and second members of the proximity detection pair.

Rolling Circle and Additional Amplification

Although RCA (rolling circle amplification) in combination with very sensitive detection or additional round of RCA of signal amplification will often produce measurable signals without amplification, PCR (or some alternative like NASBA) may be desirable for achieving specific detection in some cases, e.g., in some cases of an allele in genomic DNA. Thus, regions of genomic DNA containing sites of polymorphisms can be amplified by PCR prior to contact with circular templates. After PCR the unincorporated primers and dNTPs can be destroyed enzymatically (exonuclease and shrimp alkaline phosphatase). The enzymes would then be destroyed by heating at 80° C.

PCR in Continuous Gel Films and RCA Probes for Polymorphism Detection

SNP analysis of a large number of polymorphisms in a biallelic SNP map will sometimes require a large number of amplification reactions. Amplification, e.g., PCR (or NASBA) can be performed continuous gel arrays with RCA probes. In this case the probe is annealed to the immobilized amplification product in the gel.

Probes for both (all four) alleles for all polymorphism sites on the array are applied to the array. The probes contain allele-specific tags, of which there are a total of only four - one for each base A, C, G, T. Competing pentamers are not used, since both (all four) alleles are present during the hybridization and ligation. Presence of a restriction site is not necessary. In fact, it is unnecessary, since small fragments could diffuse from the gel arrays.

Only non-fluorescent dNTPs are present during the RCA reaction. The RCA products are labeled with generic allele-specific hybridization probes labeled with different

color fluorophors, of which there are only four (A, C, G, T). The sequences of the allele-specific tags and the probes obviously can be designed to provide very unambiguous differentiation of the four possible alleles, provided care is taken that the four fluorescent dyes are adequately separated. In this case there is great flexibility in the labeling of the probes (compared to the use of fluorescent ddNTP terminators).

Identification of RNA (RNA Profiling) and Sequencing of Mutations and SNPs Using Rolling Circle Amplification and Cantor Capture Arrays

A pre-formed circular vector can be applied to single-stranded cDNA in order to identify and quantitate the RNA molecules in a population of RNA molecules obtained from normal and disease cells. A population of circular vectors is applied to continuous gel arrays containing cDNA or RNA. The circular vectors include:

(1) A region of random DNA sequence (e.g., 5-50 bases, preferably 12 bases);

(2) A region containing a recognition sequence for a type IIS restriction enzyme that cleaves in the middle of the region of random DNA sequence (note: this region may be designed to form a hairpin or other structure as described in 5,714,320);

(3) Additional DNA sequence that is, ideally, not complementary to any of the target nucleic acid sequences (RNA or cDNA) such that the complete vector contains between 50-1500 bases.

Those circular vectors that recognize sequences in the target are separated from the population of circular vectors added to the target nucleic acids. Background hybridization can be minimized by including linear DNA that

contains all of the vector sequence except for the region of random DNA. The isolated circular vectors are amplified using rolling circle amplification (e.g., in the presence of a fluorescent nucleotides), the DNA is cleaving, e.g., using a restriction enzyme, and the resulting fragments are analyzed, e.g., interrogated on an indexing linker array (if dsDNA).

In another embodiment, circular vectors (as above) are used to identify the presence of mutations and SNPs by having a region of the circular DNA complementary to a mutation or SNP such that the circular DNA specifically binds to the mutation or SNP. Circular vectors complementary to a mutation or SNP will be isolated through application to a population of target DNA molecules (cDNA or RNA) e.g., bound to a continuous film array. The target DNA can be present as either an ordered array of distinct molecules, or as a non-ordered array of molecules on a continuous film array. The resulting vectors are amplified by rolling circle amplification (e.g., in the presence of a fluorescent nucleotides), can be fragmented by restriction enzymes, and are analyzed, e.g., on an Indexing Linker (if dsDNA) see, e.g., 5,508,169 or a Cantor array (if ssDNA) see, e.g., 5,503,980.

Vectors can be separated into pools to prevent hybridization between the vectors (dsDNA probes should be avoided) and to maximize hybridization fidelity in any method described herein. The vector pools are applied to anchored target nucleic acid (genomic DNA, amplified DNA, cDNA or RNA) and those that hybridize to sequences in the target nucleic acid are isolated from the pool (conditions selected that maximize hybridization fidelity for each vector pool). The identity of the isolated vectors is determined by RCA, where the isolated oligo probes act as

both a "positioning oligo" and an RCA primer (see 5,714,320). The DNA derived from rolling circle amplification (in the presence of a fluorescent nucleotides) is cleaved using a restriction enzyme, and the resulting fragments can be interrogated on an Indexing Linker array (if dsDNA) see, e.g., U.S. Patent No. 5,508,169 or a Cantor array (if ssDNA) see. e.g., U.S. Patent No.5,503,980.

DNA Sequencing

A linear DNA vector probe is designed with two, random, e.g., 5mer, sequences in either end of the vector. There are 1024 possible 5mer sequences, so this would entail the synthesis of 1,048,576 linear vectors. The vectors will share one or a small number of common backbones, where each backbone can include a type IIS restriction site and a priming site for DNA synthesis. The vectors should be grouped such that the random 5mers in a given group of vectors can not be brought together by the common backbone sequence. The sequence of the target nucleic acid will then facilitate the circularization of a subset of the probe vectors, with each circularized probe vector representing a short contiguous, e.g., 10 bp, stretch of target DNA. The DNA is amplified using RCA in the presence of fluorescent nucleotides. The single-stranded product of rolling circle amplification is rendered double-stranded by the annealing of un-circularized, complementary probe vector. The dsDNA RCA product is analyzed. It can be fragmented, e.g., using a type IIS restriction enzyme such that the DNA is cleaved in the middle of the short region generated by the ligation reaction. The dsDNA fragments generated by the restriction digest are analyzed, e.g., on an array of indexing linkers (see, e.g., U.S. Patent No. 5,508,169). If the probe vector is labeled with a capture moiety, e.g., biotin group, then

it is possible to render the dsDNA fragments generated from fragmentation of the RCA product single-stranded by thermal denaturation following the addition of capture moiety reactive, e.g., substrate, e.g., strepavidin-labeled substrate, e.g., magnetic beads or solid support. The single-stranded DNA fragments can then be analyzed on a Cantor array. The DNA sequence of the target DNA is reconstructed using overlap analysis according to the procedure of Drmanac et al. (see, e.g., 5,525,464; 5,492,806; 5,202,231; 5,695,940).

Example 1

Oligonucleotide probe molecules were attached to an polyacrylamide gel as described in Proudnikov et al., Anal. Biochem. 259:34-41, 1998; Timofeev et al., NAR 24:3142-48, 1996. Briefly, a continuous slab of polyacrylamide gel about 20μm thick was formed on a glass microscope slide by polymerization between the glass slide and a glass plate. The monomer N-(2,2dimtheyoxy)ethyl acrylamide was incorporated into the polyacrylamide matrix during polymerization. Before oligonucleotides were dispensed, the gel matrix was activated by incubation with aqueous trifluroacetic acid to convert diol groups in the gel to aldehyde groups. The gel was then dried.

A total of 20 drops of amine-derivatized oligonucleotides labeled with ^{32}-P were dispensed onto the surface of the dried gel. The distribution of the labeled oligonucleotide an a dried acrylamide slab was examined by phosphor imaging and found to consists of a series of discrete, resolved spots.

Example 2

A ^{32}-P labeled oligonucleotide sample was applied using a piezo dispenser as described in Example 1. The continuous polyacrylamide matrix was placed under a layer of chloroform which, in turn, was overlaid with water containing a pyridine-borane complex. Water was able to diffuse through the chloroform layer to hydrate the gel, but the chloroform prevented diffusion of the oligonucleotide out of the gel. Schiff bases that formed between the amine groups on the oligonucleotides and the aldehyde groups with the gel were reduced by the pyridine-borane complex, which also diffused through the chloroform layer to covalently link the oligonucleotide probe molecules to the acrylamide matrix. Phosphor imaging of ^{32}-labeled oligonucleotides after reducing and washing the matrix revealed that the oligonucleotide remained in discrete spots, similar to the pattern observed on polyacrylamide substrates that was not processed after dispensing as described in Example 1. Quantitation revealed that 80 percent of the oligonucleotide was retained in the matrix.

Example 3

A ^{32}-labeled oligonucleotide was applied to a gel matrix as described in Example 1, except that the slide was simply washed with sodium borohydride, a reducing agent, after dispensing. The covalently immobilization step was also omitted. Under these conditions, approximately 80% of the oligonucleotide was washed out of the matrix. These results demonstrate that an linking step is required to retain the oligonucleotides within the gel matrix.

It is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate

and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

What is claimed is:

1. A continuous surface substrate comprising an array of polymers having diverse sequences.

2. The continuous surface of claim 1, wherein the surface substrate comprises polyacrylamide.

3. The continuous surface of claim 1, wherein the continuous surface comprises agarose.

4. The continuous surface of claim 2 wherein the pored substrate is a two dimensional array of molecules, wherein each of the molecules is positionally distinguishable from other molecules of the plurality on the array, and wherein each positional distinguishable molecules is different form the others.

5. A method of analyzing a test nucleotide in a polynucleotide, comprising:

(1) providing a sample which includes a sample polynucleotide sequence, which includes a test nucleotide, to be analyzed;

(2) (a) annealing an effective amount of sample polynucleotide sequence to a single-stranded circular template, wherein the single-stranded circular template includes at least one copy of a nucleotide sequence complementary to the sequence of the sample polynucleotide sequence and optionally,

(b) combining the circular template with an effective amount of at least two types of nucleotide triphosphates and an effective amount of a polymerase enzyme to yield a product, e.g., a single-stranded oligonucleotide multimer complementary to the circular oligonucleotide template; and optionally

cleaving the product to produce cleaved amplified product, and

(3) analyzing said product from (2) b or c, e.g., on a two dimensional pored array.

CONTINUOUS SURFACE ARRAYS

Abstract of the Disclosure

Arrays of polymers on continuous surfaces and methods of making and using same are disclosed.